

1                   **FIBRINOGEN BIOPOLYMER MARKER**  
2                   **PREDICTIVE OF TYPE II DIABETES**  
3

4    FIELD OF THE INVENTION

5           This invention relates to the field of characterizing  
6    the existence of a disease state; particularly to the  
7    utilization of mass spectrometry to elucidate particular  
8    biopolymer markers indicative or predictive of a particular  
9    disease state, and most particularly to specific biopolymer  
10   markers whose up-regulation, down-regulation, or relative  
11   presence in disease vs. normal states has been determined to  
12   be useful in disease state assessment and therapeutic target  
13   recognition, development and validation.

14   BACKGROUND OF THE INVENTION

15           Methods utilizing mass spectrometry for the analysis of  
16   a target polypeptide have been taught wherein the polypeptide  
17   is first solubilized in an appropriate solution or reagent  
18   system. The type of solution or reagent system, e.g.,  
19   comprising an organic or inorganic solvent, will depend on  
20   the properties of the polypeptide and the type of mass  
21   spectrometry performed and are well-known in the art (see,  
22   e.g., Vorm et al. (1994) Anal. Chem. 66:3281 (for MALDI) and  
23   Valaskovic et al. (1995) Anal. Chem. 67:3802 (for ESI). Mass  
24   spectrometry of peptides is further disclosed, e.g., in WO

1 93/24834 by Chait et al.

2 In one prior art embodiment, the solvent is chosen so  
3 that the risk that the molecules may be decomposed by the  
4 energy introduced for the vaporization process is  
5 considerably reduced, or even fully excluded. This can be  
6 achieved by embedding the sample in a matrix, which can be an  
7 organic compound, e.g., sugar, in particular pentose or  
8 hexose, but also polysaccharides such as cellulose. These  
9 compounds are decomposed thermolytically into CO<sub>2</sub> and H<sub>2</sub>O so  
10 that no residues are formed which might lead to chemical  
11 reactions. The matrix can also be an inorganic compound,  
12 e.g., nitrate of ammonium which is decomposed practically  
13 without leaving any residues. Use of these and other solvents  
14 are further disclosed in U.S. Pat. No. 5,062,935 by Schlag et  
15 al.

16 Prior art mass spectrometer formats for use in analyzing  
17 the translation products include ionization (I) techniques,  
18 including but not limited to matrix assisted laser desorption  
19 (MALDI), continuous or pulsed electrospray (ESI) and related  
20 methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster  
21 impact (MCI); these ion sources can be matched with detection  
22 formats including linear or non-linear reflection time-of-  
23 flight (TOF), single or multiple quadropole, single or

multiple magnetic sector, Fourier Transform ion cyclotron resonance (FTICR), ion trap, and combinations thereof (e.g., ion-trap/time-of-flight). For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed. Subattomole levels of protein have been detected, for example, using ESI (Valaskovic, G. A. et al., (1996) Science 273:1199-1202) or MALDI (Li, L. et al., (1996) J. Am. Chem. Soc. 118:1662-1663) mass spectrometry.

ES mass spectrometry has been introduced by Fenn et al. (J. Phys. Chem. 88, 4451-59 (1984); PCT Application No. WO 90/14148) and current applications are summarized in recent review articles (R. D. Smith et al., Anal. Chem. 62, 882-89 (1990) and B. Ardrey, Electrospray Mass Spectrometry, Spectroscopy Europe, 4, 10-18 (1992)). MALDI-TOF mass spectrometry has been introduced by Hillenkamp et al. ("Matrix Assisted UV-Laser Desorption/Ionization: A New Approach to Mass Spectrometry of Large Biomolecules," Biological Mass Spectrometry (Burlingame and McCloskey, editors), Elsevier Science Publishers, Amsterdam, pp. 49-60, 1990). With ESI, the determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks which all could be used for

1 the mass calculation.

2 The mass of the target polypeptide determined by mass  
3 spectrometry is then compared to the mass of a reference  
4 polypeptide of known identity. In one embodiment, the target  
5 polypeptide is a polypeptide containing a number of repeated  
6 amino acids directly correlated to the number of  
7 trinucleotide repeats transcribed/translated from DNA; from  
8 its mass alone the number of repeated trinucleotide repeats  
9 in the original DNA which coded it, may be deduced.

10 U.S. Patent No. 6,020,208 utilizes a general category of  
11 probe elements (i.e., sample presenting means) with Surfaces  
12 Enhanced for Laser Desorption/Ionization (SELDI), within  
13 which there are three (3) separate subcategories. The SELDI  
14 process is directed toward a sample presenting means (i.e.,  
15 probe element surface) with surface-associated (or surface-  
16 bound) molecules to promote the attachment (tethering or  
17 anchoring) and subsequent detachment of tethered analyte  
18 molecules in a light-dependent manner, wherein the said  
19 surface molecule(s) are selected from the group consisting of  
20 photoactive (photolabile) molecules that participate in the  
21 binding (docking, tethering, or crosslinking) of the analyte  
22 molecules to the sample presenting means (by covalent  
23 attachment mechanisms or otherwise).

1 PCT/EP/04396 teaches a process for determining the  
2 status of an organism by peptide measurement. The reference  
3 teaches the measurement of peptides in a sample of the  
4 organism which contains both high and low molecular weight  
5 peptides and acts as an indicator of the organism's status.  
6 The reference concentrates on the measurement of low  
7 molecular weight peptides, i.e. below 30,000 Daltons, whose  
8 distribution serves as a representative cross-section of  
9 defined controls. Contrary to the methodology of the instant  
10 invention, the '396 patent strives to determine the status of  
11 a healthy organism, i.e. a "normal" and then use this as a  
12 reference to differentiate disease states. The present  
13 inventors do not attempt to develop a reference "normal", but  
14 rather strive to specify particular markers whose presence,  
15 absence or relative strength/concentration in disease vs.  
16 normal is diagnostic of at least one specific disease state  
17 or whose up-regulation or down-regulation is predictive of at  
18 least one specific disease state, whereby the presence of  
19 said marker serves as a positive indicator useful in  
20 distinguishing disease state. This leads to a simple method  
21 of analysis which can easily be performed by an untrained  
22 individual, since there is a positive correlation of data.  
23 On the contrary, the '396 patent requires a complicated

1 analysis by a highly trained individual to determine disease  
2 state versus the perception of non-disease or normal  
3 physiology.

4 Richter et al, Journal of Chromatography B, 726(1999)  
5 25-35, refer to a database established from human  
6 hemofiltrate comprised of a mass database and a sequence  
7 database. The goal of Richter et al was to analyze the  
8 composition of the peptide fraction in human blood. Using  
9 MALDI-TOF, over 20,000 molecular masses were detected  
10 representing an estimated 5,000 different peptides. The  
11 conclusion of the study was that the hemofiltrate (HF)  
12 represented the peptide composition of plasma. No  
13 correlation of peptides with relation to normal and/or  
14 disease states is made.

15 As used herein, "analyte" refers to any atom and/or  
16 molecule; including their complexes and fragment ions. The  
17 term may refer to a single component or a set of components.  
18 In the case of biological molecules/macromolecules or  
19 "biopolymers", such analytes include but are not limited to:  
20 polypeptides, polynucleotides, proteins, peptides,  
21 antibodies, DNA, RNA, carbohydrates, steroids, and lipids,  
22 and any detectable moiety thereof, e.g. immunologically  
23 detectable fragments. Note that most important biomolecules

1 under investigation for their involvement in the structure or  
2 regulation of life processes are quite large (typically  
3 several thousand times larger than H<sub>2</sub>O).

4 As used herein, the term "molecular ions" refers to  
5 molecules in the charged or ionized state, typically by the  
6 addition or loss of one or more protons (H<sup>+</sup>).

7 As used herein, the term "molecular fragmentation" or  
8 "fragment ions" refers to breakdown products of analyte  
9 molecules caused, for example, during laser-induced  
10 desorption (especially in the absence of added matrix).

11 As used herein, the term "solid phase" refers to the  
12 condition of being in the solid state, for example, on the  
13 probe element surface.

14 As used herein, "gas" or "vapor phase" refers to  
15 molecules in the gaseous state (i.e., in vacuo for mass  
16 spectrometry).

17 As used herein, the term "analyte desorption/ionization"  
18 refers to the transition of analytes from the solid phase to  
19 the gas phase as ions. Note that the successful  
20 desorption/ionization of large, intact molecular ions by  
21 laser desorption is relatively recent (circa 1988)--the big  
22 breakthrough was the chance discovery of an appropriate  
23 matrix (nicotinic acid).

1 As used herein, the term "gas phase molecular ions"  
2 refers to those ions that enter into the gas phase. Note that  
3 large molecular mass ions such as proteins (typical  
4 mass=60,000 to 70,000 times the mass of a single proton) are  
5 typically not volatile (i.e., they do not normally enter into  
6 the gas or vapor phase). However, in the procedure of the  
7 present invention, large molecular mass ions such as proteins  
8 do enter the gas or vapor phase.

9 As used herein in the case of MALDI, the term "matrix"  
10 refers to any one of several small, acidic, light absorbing  
11 chemicals (e.g., CHCA (alpha-cyano-4-hydroxy-cinnamic acid),  
12 nicotinic or sinapinic acid) that is mixed in solution with  
13 the analyte in such a manner so that, upon drying on the  
14 probe element, the crystalline matrix-embedded analyte  
15 molecules are successfully desorbed (by laser irradiation)  
16 and ionized from the solid phase (crystals) into the gaseous  
17 or vapor phase and accelerated as intact molecular ions. For  
18 the MALDI process to be successful, analyte is mixed with a  
19 freshly prepared solution of the chemical matrix (e.g.,  
20 10,000:1 matrix:analyte) and placed on the inert probe  
21 element surface to air dry just before the mass spectrometric  
22 analysis. The large fold molar excess of matrix, present at  
23 concentrations near saturation, facilitates crystal formation

1 and entrapment of analyte.

2 As used herein, "energy absorbing molecules (EAM)"  
3 refers to any one of several small, light absorbing chemicals  
4 that, when presented on the surface of a probe, facilitate  
5 the neat desorption of molecules from the solid phase (i.e.,  
6 surface) into the gaseous or vapor phase for subsequent  
7 acceleration as intact molecular ions. The term EAM is  
8 preferred, especially in reference to SELDI. Note that  
9 analyte desorption by the SELDI process is defined as a  
10 surface-dependent process (i.e., neat analyte may be placed  
11 on a surface composed of bound EAM or EAM and analyte may be  
12 mixed prior to placement on a surface). In contrast, MALDI is  
13 presently thought to facilitate analyte desorption by a  
14 volcanic eruption-type process that "throws" the entire  
15 surface into the gas phase. Furthermore, note that some EAM  
16 when used as free chemicals to embed analyte molecules as  
17 described for the MALDI process will not work (i.e., they do  
18 not promote molecular desorption, thus they are not suitable  
19 matrix molecules).

20 As used herein, "probe element" or "sample presenting  
21 device" refers to an element having the following properties:  
22 it is inert (for example, typically stainless steel) and  
23 active (probe elements with surfaces enhanced to contain EAM

1 and/or molecular capture devices).

2 As used herein, "MALDI" refers to Matrix-Assisted Laser  
3 Desorption/Ionization.

4 As used herein, "TOF" stands for Time-of-Flight.

5 As used herein, "MS" refers to Mass Spectrometry.

6 As used herein, "MS/MS" refers to multiple sequential  
7 mass spectrometry.

8 As used herein "MALDI-TOF MS" refers to Matrix-assisted  
9 laser desorption/ionization time-of-flight mass spectrometry.

10 As used herein, "ESI" is an abbreviation for  
11 electrospray ionization.

12 As used herein, "chemical bonds" is used simply as an  
13 attempt to distinguish a rational, deliberate, and  
14 knowledgeable manipulation of known classes of chemical  
15 interactions from the poorly defined kind of general  
16 adherence observed when one chemical substance (e.g., matrix)  
17 is placed on another substance (e.g., an inert probe element  
18 surface). Types of defined chemical bonds include  
19 electrostatic or ionic (+/-) bonds (e.g., between a  
20 positively and negatively charged groups on a protein  
21 surface), covalent bonds (very strong or "permanent" bonds  
22 resulting from true electron sharing), coordinate covalent  
23 bonds (e.g., between electron donor groups in proteins and

1 transition metal ions such as copper or iron), and  
2 hydrophobic interactions (such as between two noncharged  
3 groups), weak dipole and London force or induced dipole  
4 interactions.

5 As used herein, "electron donor groups" refers to the  
6 case of biochemistry, where atoms in biomolecules (e.g, N, S,  
7 O) "donate" or share electrons with electron poor groups  
8 (e.g., Cu ions and other transition metal ions).

9 As used herein, the term "biopolymer markers indicative  
10 or predictive of a disease state" is interpreted to mean that  
11 a biopolymer marker which is strongly present in a normal  
12 individual, but is down-regulated in disease is predictive of  
13 said disease; while alternatively, a biopolymer marker which  
14 is strongly present in a disease state, but is down-regulated  
15 in normal individuals, is indicative of said disease state.  
16 Biopolymer markers which are present in both disease and  
17 normal states are indicative/predictive based upon their  
18 relative strengths in disease vs. normal, along with the  
19 observation regarding when their signal strengthens/weakens  
20 relative to disease manifestation or progression.

21 As used herein, the term "disease state assessment" is  
22 interpreted to mean quantitative or qualitative determination  
23 of the presence/absence of the disease, with or without an

1 ability to determine severity, rapidity of onset, or  
2 resolution of the disease state, e.g. a return to a normal  
3 physiological state.

4 As used herein, the term "therapeutic target  
5 recognition, development, and validation" refers to any  
6 concept or method which enables an artisan to recognize,  
7 develop, or validate the efficacy of a therapeutic moiety  
8 which is effected in conjunction with a chemical or physical  
9 interaction with one or more of the biopolymer markers of the  
10 instant invention.

11 As used herein, the term "polypeptide" is interpreted to  
12 mean a polymer composed of amino acid residues, related  
13 naturally occurring structural variants, and synthetic non-  
14 naturally occurring analogs thereof linked via peptide bonds,  
15 related naturally occurring structural variants, and  
16 synthetic non-naturally occurring analogs thereof. Synthetic  
17 polypeptides can be synthesized, for example, using an  
18 automated polypeptide synthesizer. The term "protein"  
19 typically refers to large polypeptides. The term "peptide"  
20 typically refers to short polypeptides. "Polypeptide(s)"  
21 refers to any peptide or protein comprising two or more amino  
22 acids joined to each other by peptide bonds or modified  
23 peptide bonds. "Polypeptide(s)" refers to both short chains,

commonly referred to as peptides, oligopeptides and oligomers  
and to longer chains generally referred to as proteins.  
Polypeptides may contain amino acids other than the 20 gene  
encoded amino acids. "Polypeptide(s)" include those modified  
either by natural processes, such as processing and other  
post-translational modifications, but also by chemical  
modification techniques. Such modifications are well  
described in basic texts and in more detailed monographs, as  
well as in a voluminous research literature, and they are  
well-known to those of skill in the art. It will be  
appreciated that the same type of modification may be present  
in the same or varying degree at several sites in a given  
polypeptide. Also, a given polypeptide may contain many types  
of modifications. Modifications can occur anywhere in a  
polypeptide, including the peptide backbone, the amino acid  
side-chains, and the amino or carboxyl termini. Modifications  
include, for example, acetylation, acylation, ADP-  
ribosylation, amidation, covalent attachment of flavin,  
covalent attachment of a heme moiety, covalent attachment of  
a nucleotide or nucleotide derivative, covalent attachment of  
a lipid or lipid derivative, covalent attachment of  
phosphatidylinositol, cross-linking, cyclization, disulfide  
bond formation, demethylation, formation of covalent cross-

1 links, formation of cysteine, formation of pyroglutamate,  
2 formylation, gamma-carboxylation, glycosylation, GPI anchor  
3 formation, hydroxylation, iodination, methylation,  
4 myristoylation, oxidation, proteolytic processing,  
5 phosphorylation, prenylation, racemization, glycosylation,  
6 lipid attachment, sulfation, gamma-carboxylation of glutamic  
7 acid residues, hydroxylation and ADP-ribosylation,  
8 selenoylation, sulfation, transfer-RNA mediated addition of  
9 amino acids to proteins, such as arginylation, and  
10 ubiquitination. See, for instance, PROTEINS--STRUCTURE AND  
11 MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman  
12 and Company, New York (1993) and Wold, F., Posttranslational  
13 Protein Modifications: Perspectives and Prospects, pgs. 1-12  
14 in POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C.  
15 Johnson, Ed., Academic Press, New York (1983); Seifter et  
16 al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al.,  
17 Protein Synthesis: Posttranslational Modifications and Aging,  
18 Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be  
19 branched or cyclic, with or without branching. Cyclic,  
20 branched and branched circular polypeptides may result from  
21 post-translational natural processes and may be made by  
22 entirely synthetic methods, as well.

23 As used herein, the term "polynucleotide" is interpreted

1 to mean a polymer composed of nucleotide units.

2 Polynucleotides include naturally occurring nucleic acids,  
3 such as deoxyribonucleic acid ("DNA") and ribonucleic acid  
4 ("RNA") as well as nucleic acid analogs. Nucleic acid analogs  
5 include those which include non-naturally occurring bases,  
6 nucleotides that engage in linkages with other nucleotides  
7 other than the naturally occurring phosphodiester bond or  
8 which include bases attached through linkages other than  
9 phosphodiester bonds. Thus, nucleotide analogs include, for  
10 example and without limitation, phosphorothioates,  
11 phosphorodithioates, phosphorotriesters,  
12 phosphoramidates, boranophosphates, methylphosphonates,  
13 chiral-methyl phosphonates, 2-O-methyl ribonucleotides,  
14 peptide-nucleic acids (PNAs), and the like. Such  
15 polynucleotides can be synthesized, for example, using an  
16 automated DNA synthesizer. The term "nucleic acid" typically  
17 refers to large polynucleotides. The term "oligonucleotide"  
18 typically refers to short polynucleotides, generally no  
19 greater than about 50 nucleotides. It will be understood that  
20 when a nucleotide sequence is represented by a DNA sequence  
21 (i.e., A, T, G, C), this also includes an RNA sequence (i.e.,  
22 A, U, G, C) in which "U" replaces T.

23 As used herein, the term "detectable moiety" or a

1 "label" refers to a composition detectable by spectroscopic,  
2 photochemical, biochemical, immunochemical, or chemical  
3 means. For example, useful labels include <sup>32</sup>P, <sup>35</sup>S,  
4 fluorescent dyes, electron-dense reagents, enzymes (e.g., as  
5 commonly used in an ELISA), biotin-streptavidin, dioxigenin,  
6 haptens and proteins for which antisera or monoclonal  
7 antibodies are available, or nucleic acid molecules with a  
8 sequence complementary to a target. The detectable moiety  
9 often generates a measurable signal, such as a radioactive,  
10 chromogenic, or fluorescent signal, that can be used to  
11 quantitate the amount of bound detectable moiety in a sample.  
12 The detectable moiety can be incorporated in or attached to a  
13 primer or probe either covalently, or through ionic, van  
14 der Waals or hydrogen bonds, e.g., incorporation of  
15 radioactive nucleotides, or biotinylated nucleotides that are  
16 recognized by streptavidin. The detectable moiety may be  
17 directly or indirectly detectable. Indirect detection can  
18 involve the binding of a second directly or indirectly  
19 detectable moiety to the detectable moiety. For example, the  
20 detectable moiety can be the ligand of a binding partner,  
21 such as biotin, which is a binding partner for streptavidin,  
22 or a nucleotide sequence, which is the binding partner for a  
23 complementary sequence, to which it can specifically

1 hybridize. The binding partner may itself be directly  
2 detectable, for example, an antibody may be itself labeled  
3 with a fluorescent molecule. The binding partner also may be  
4 indirectly detectable, for example, a nucleic acid having a  
5 complementary nucleotide sequence can be a part of a branched  
6 DNA molecule that is in turn detectable through hybridization  
7 with other labeled nucleic acid molecules. (See, e.g., P. D.  
8 Fahrlander and A. Klausner, Bio/Technology (1988) 6:1165.)  
9 Quantitation of the signal is achieved by, e.g.,  
10 scintillation counting, densitometry, or flow cytometry.

11 As used herein, the term "antibody or antibodies"  
12 includes polyclonal and monoclonal antibodies of any isotype  
13 (IgA, IgG, IgE, IgD, IgM), or an antigen-binding portion  
14 thereof, including but not limited to F(ab) and Fv fragments,  
15 single chain antibodies, chimeric antibodies, humanized  
16 antibodies, and a Fab expression library. "Antibody" refers  
17 to a polypeptide ligand substantially encoded by an  
18 immunoglobulin gene or immunoglobulin genes, or fragments  
19 thereof, which specifically binds and recognizes an epitope  
20 (e.g., an antigen). The recognized immunoglobulin --genes  
21 include the kappa and lambda light chain constant region  
22 genes, the alpha, gamma, delta, epsilon and mu heavy chain  
23 constant region genes, and the myriad immunoglobulin variable

1 region genes. Antibodies exist, e.g., as intact  
2 immunoglobulins or as a number of well characterized  
3 fragments produced by digestion with various peptidases. This  
4 includes, e.g., Fab' and F(ab)'<sub>2</sub> fragments. The term  
5 "antibody," as used herein, also includes antibody fragments  
6 either produced by the modification of whole antibodies or  
7 those synthesized de novo using recombinant DNA  
8 methodologies. It also includes polyclonal antibodies,  
9 monoclonal antibodies, chimeric antibodies and humanized  
10 antibodies. "Fc" portion of an antibody refers to that  
11 portion of an immunoglobulin heavy chain that comprises one  
12 or more heavy chain constant region domains, CH, CH<sub>2</sub> and CH<sub>3</sub>,  
13 but does not include the heavy chain variable region.

14 As used herein, the term "moieties" refers to an  
15 indefinite portion of a sample.

16 A "ligand" is a compound that specifically binds to a  
17 target molecule.

18 A "receptor" is a compound or portion of a structure  
19 that specifically binds to a ligand.

20 A ligand or a receptor (e.g., an antibody) "specifically  
21 binds to" or "is specifically immunoreactive with" a compound  
22 analyte when the ligand or receptor functions in a binding  
23 reaction which is determinative of the presence of the

1 analyte in a sample of heterogeneous compounds. Thus, under  
2 designated assay (e.g., immunoassay) conditions, the ligand  
3 or receptor binds preferentially to a particular analyte and  
4 does not bind in a significant amount to other compounds  
5 present in the sample. For example, a polynucleotide  
6 specifically binds under hybridization conditions to an  
7 analyte polynucleotide comprising a complementary sequence;  
8 an antibody specifically binds under immunoassay conditions  
9 to an antigen analyte bearing an epitope against which the  
10 antibody was raised; and an adsorbent specifically binds to  
11 an analyte under proper elution conditions.

12 As used herein, the term "pharmaceutically effective  
13 carrier" refers to any solid or liquid material which may be  
14 used in creating formulations that further include active  
15 ingredients of the instant invention, e.g. biopolymer markers  
16 or therapeutics, for administration to a patient.

17 As used herein, the term "agent" is interpreted to mean  
18 a chemical compound, a mixture of chemical compounds, a  
19 sample of undetermined composition, a combinatorial small  
20 molecule array, a biological macromolecule, a bacteriophage  
21 peptide display library, a bacteriophage antibody (e.g.,  
22 scFv) display library, a polysome peptide display library, or  
23 an extract made from biological materials such as bacteria,

1 plants, fungi, or animal cells or tissues. Suitable  
2 techniques involve selection of libraries of recombinant  
3 antibodies in phage or similar vectors. See, Huse et al.  
4 (1989) Science 246: 1275-1281; and Ward et al. (1989) Nature  
5 341: 544-546. The protocol described by Huse is  
6 rendered more efficient in combination with phage display  
7 technology. See, e.g., Dower et al., WO 91/17271 and  
8 McCafferty et al., WO 92/01047.

9 As used herein, the term "isolated" is interpreted to  
10 mean altered "by the hand of man" from its natural state,  
11 i.e., if it occurs in nature, it has been changed or removed  
12 from its original environment, or both. For example, a  
13 polynucleotide or a polypeptide naturally present in a living  
14 organism is not "isolated," but the same polynucleotide or  
15 polypeptide separated from the coexisting materials of its  
16 natural state is "isolated", as the term is employed herein.

17 As used herein, the term "variant" is interpreted to  
18 mean a polynucleotide or polypeptide that differs from a  
19 reference polynucleotide or polypeptide respectively, but  
20 retains essential properties. A typical variant of a  
21 polynucleotide differs in nucleotide sequence from another,  
22 reference polynucleotide. Changes in the nucleotide sequence  
23 of the variant may or may not alter the amino acid sequence

1 of a polypeptide encoded by the reference polynucleotide.  
2 Nucleotide changes may result in amino acid substitutions,  
3 additions, deletions, fusions and truncations in the  
4 polypeptide encoded by the reference sequence, as discussed  
5 below. A typical variant of a polypeptide differs in amino  
6 acid sequence from another, reference polypeptide. Generally,  
7 differences are limited so that the sequences of the  
8 reference polypeptide and the variant are closely similar  
9 overall and, in many regions, identical. A variant and  
10 reference polypeptide may differ in amino acid sequence by  
11 one or more substitutions, additions, deletions in any  
12 combination. A substituted or inserted amino acid residue may  
13 or may not be one encoded by the genetic code. A variant of a  
14 polynucleotide or polypeptide may be a naturally occurring  
15 such as an allelic variant, or it may be a variant that is  
16 not known to occur naturally. Non-naturally occurring  
17 variants of polynucleotides and polypeptides may be made by  
18 mutagenesis techniques, by direct synthesis, and by other  
19 recombinant methods known to skilled artisans.

20 As used herein, the term "biopolymer marker" refers to a  
21 polymer of biological origin, e.g. polypeptides,  
22 polynucleotides, polysaccharides or polyglycerides (e.g., di-  
23 or tri-glycerides), and may include any fragment, e.g.

1 immunologically reactive fragments, variants or moieties  
2 thereof.

3 As used herein, the term "fragment" refers to the  
4 products of the chemical, enzymatic, or physical breakdown of  
5 an analyte. Fragments may be in a neutral or ionic state.

6 As used herein, the term "therapeutic avenues" is  
7 interpreted to mean any agents, modalities, synthesized  
8 compounds, etc., which interact with a biopolymer marker in  
9 any manner that facilitates a therapeutic benefit, including  
10 immunotherapeutic intervention, e.g. modalities such as  
11 administration of an immunologically reactive moiety capable  
12 of altering the course, progression and/or manifestation of  
13 the disease, as a result of interfering with the disease  
14 manifestation process, for example, at the early stages  
15 focused upon by the identification of the disease, such as by  
16 supplying a moiety capable of modifying the pathogenicity of  
17 lymphocytes specific for the biopolymer marker or related  
18 components.

19 As used herein, the term "interacting with a biopolymer  
20 marker" includes any process by which a biopolymer marker may  
21 physically or chemically relate with an organism,  
22 particularly when this interaction results in the development  
23 of therapeutic avenues or in modulation of the disease state.

As used herein, the term "therapeutic targets" may thus be defined as those analytes which are capable of exerting a modulating force, wherein "modulation" is defined as an alteration in function inclusive of activity, synthesis, production, and circulating levels. Thus, modulation effects the level or physiological activity of at least one particular disease related biopolymer marker or any compound or biomolecule whose presence, level or activity is linked either directly or indirectly, to an alteration of the presence, level, activity or generic function of the biopolymer marker, and may include pharmaceutical agents, biomolecules that bind to the biopolymer markers, or biomolecules or complexes to which the biopolymer markers bind. The binding of the biopolymer markers and the therapeutic moiety may result in activation (agonist), inhibition (antagonist), or an increase or decrease in activity or production (modulator) of the biopolymer markers or the bound moiety. Examples of such therapeutic moieties include, but are not limited to, antibodies, oligonucleotides, proteins (e.g., receptors), RNA, DNA, enzymes, peptides or small molecules. With regard to immunotherapeutic moieties, such a moiety may be defined as an effective analog for a major epitope peptide which has the

1 ability to reduce the pathogenicity of key lymphocytes which  
2 are specific for the native epitope. An analog is defined as  
3 having structural similarity but not identity in peptide  
4 sequencing able to be recognized by T-cells spontaneously  
5 arising and targeting the endogeneous self epitope. A  
6 critical function of this analog is an altered T-cell  
7 activation which leads to T-cell anergy or death.

8 With the advent of mass spectrometric methods such as  
9 MALDI and SELDI and ESI, researchers have begun to utilize a  
10 tool that holds the promise of uncovering countless  
11 biopolymers which result from translation, transcription and  
12 post-translational transcription of proteins from the entire  
13 genome.

14 Operating upon the principles of retentate  
15 chromatography, SELDI MS involves the adsorption of proteins,  
16 based upon their physico-chemical properties at a given pH  
17 and salt concentration, followed by selectively desorbing  
18 proteins from the surface by varying pH, salt, or organic  
19 solvent concentration. After selective desorption, the  
20 proteins retained on the SELDI surface, the "chip", can be  
21 analyzed using the CIPHERGEN protein detection system, or an  
22 equivalent thereof. Retentate chromatography is limited,  
23 however, by the fact that if unfractionated body fluids, e.g.

1 blood, blood products, urine, saliva, cerebrospinal fluid,  
2 lymph and the like, along with tissue samples, are applied  
3 to the adsorbent surfaces, the biopolymers present in the  
4 greatest abundance will compete for all the available binding  
5 sites and thereby prevent or preclude less abundant  
6 biopolymers from interacting with them, thereby reducing or  
7 eliminating the diversity of biopolymers which are readily  
8 ascertainable.

9 If a process could be devised for maximizing the  
10 diversity of biopolymers discernable from a sample, the  
11 ability of researchers to accurately determine the relevance  
12 of such biopolymers with relation to one or more disease  
13 states would be immeasurably enhanced.

#### 14 15 SUMMARY OF THE INVENTION

16 The instant invention is characterized by the use of a  
17 combination of preparatory steps, e.g. chromatography and 1-D  
18 tricine polyacrylamide gel electrophoresis. Subsequent to  
19 which the gel is stained, e.g. with Coomassie blue, silver or  
20 rubidium. Next, bands are selected from the gels for further  
21 study. Tryptic digestion of each band follows, concluding  
22 with the extraction of tryptic peptides from the digest.  
23 This extraction may be accomplished utilizing C18 ZIPTIPs, or

1 organic extract and dry technique followed by MALDI Qq TOF  
2 (Maldi Quadrupole Quadrupole Time of Flight) processing.

3 Additional methodologies may include SELDI MS, 2-D gel  
4 technology, MALDI MS/MS and time-of-flight detection  
5 procedures to maximize the diversity of biopolymers which are  
6 verifiable within a particular sample. The cohort of  
7 biopolymers verified within a sample is then compared to  
8 develop data indicating their presence, absence or relative  
9 strength/concentration in disease vs normal controls, and  
10 further studied to determine whether the up-regulation or  
11 down-regulation of a single biopolymer or group of  
12 biopolymers is indicative of a disease state or predictive of  
13 the development of said disease state. Additionally,  
14 biopolymers recognized as being indicative or predictive of a  
15 disease state in accordance with the instant invention are  
16 useful in therapeutic intervention, e.g. as therapeutic  
17 modalities in their own right, in the course of therapeutic  
18 target recognition, in the development and validation of  
19 efficacious therapeutic modalities, e.g when interrogating or  
20 developing phage display libraries, and as ligands or  
21 receptors for use in conjunction with therapeutic  
22 intervention.

23 Although all manner of biomarkers related to all disease

1 conditions are deemed to be within the purview of the instant  
2 invention and methodology, particular significance was given  
3 to those markers and diseases associated with the complement  
4 system, cognitive diseases, e.g. Alzheimer's disease and  
5 Syndrome X and diseases related thereto.

6 The complement system is an important part of non-clonal  
7 or innate immunity that collaborates with acquired immunity  
8 to destroy invading pathogens and to facilitate the clearance  
9 of immune complexes from the system. This system is the  
10 major effector of the humoral branch of the immune system,  
11 consisting of nearly 30 serum and membrane proteins. The  
12 proteins and glycoproteins composing the complement system  
13 are synthesized largely by liver hepatocytes. Activation of  
14 the complement system involves a sequential enzyme cascade in  
15 which the proenzyme product of one step becomes the enzyme  
16 catalyst of the next step. Complement activation can occur  
17 via two pathways: the classical and the alternative. The  
18 classical pathway is commonly initiated by the formation of  
19 soluble antigen-antibody complexes or by the binding of  
20 antibody to antigen on a suitable target, such as a bacterial  
21 cell. The alternative pathway is generally initiated by  
22 various cell-surface constituents that are foreign to the  
23 host. Each complement component is designated by numerals

1 (C1-C9), by letter symbols, or by trivial names. After a  
2 component is activated, the peptide fragments are denoted by  
3 small letters. The complement fragments interact with one  
4 another to form functional complexes. Ultimately, foreign  
5 cells are destroyed through the process of a membrane-attack  
6 complex mediated lysis.

7 The C4 component of the complement system is involved in  
8 the classical activation pathway. It is a glycoprotein  
9 containing three polypeptide chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). C4 is a  
10 substrate of component C1s and is activated when C1s  
11 hydrolyzes a small fragment (C4a) from the amino terminus of  
12 the  $\alpha$  chain, exposing a binding site on the larger fragment  
13 (C4b).

14 The native C3 component consists of two polypeptide  
15 chains,  $\alpha$  and  $\beta$ . As a serum protein, C3 is involved in the  
16 alternative pathway. Serum C3, which contains an unstable  
17 thioester bond, is subject to slow spontaneous hydrolysis  
18 into C3a and C3b. The C3f component is involved in the  
19 regulation required of the complement system which confines  
20 the reaction to designated targets. During the regulation  
21 process, C3b is cleaved into two parts: C3bi and C3f. C3bi  
22 is a membrane-bound intermediate wherein C3f is a free  
23 diffusible (soluble) component.

1 Complement components have been implicated in the  
2 pathogenesis of several disease conditions. C3 deficiencies  
3 have the most severe clinical manifestations, such as  
4 recurrent bacterial infections and immune-complex diseases,  
5 reflecting the central role of C3. The rapid profusion of  
6 C3f moieties and resultant "accidental" lysis of normal cells  
7 mediated thereby gives rise to a host of auto-immune  
8 reactions. The ability to understand and control these  
9 mechanisms, along with their attendant consequences, will  
10 enable practitioners to develop both diagnostic and  
11 therapeutic avenues by which to thwart these maladies.

12 In the course of defining a plurality of disease  
13 specific marker sequences, special significance was given to  
14 markers which were evidentiary of a particular disease state  
15 or with conditions associated with Syndrome-X. Syndrome-X is  
16 a multifaceted syndrome, which occurs frequently in the  
17 general population. A large segment of the adult population  
18 of industrialized countries develops this metabolic syndrome,  
19 produced by genetic, hormonal and lifestyle factors such as  
20 obesity, physical inactivity and certain nutrient excesses.  
21 This disease is characterized by the clustering of insulin  
22 resistance and hyperinsulinemia, and is often associated with  
23 dyslipidemia (atherogenic plasma lipid profile), essential

1 hypertension, abdominal (visceral) obesity, glucose  
2 intolerance or noninsulin-dependent diabetes mellitus and an  
3 increased risk of cardiovascular events. Abnormalities of  
4 blood coagulation (higher plasminogen activator inhibitor  
5 type I and fibrinogen levels), hyperuricemia and  
6 microalbuminuria have also been found in metabolic syndrome-  
7 X.

8 The instant inventors view the Syndrome X continuum in  
9 its cardiovascular light, while acknowledging its important  
10 metabolic component. The first stage of Syndrome X consists  
11 of insulin resistance, abnormal blood lipids (cholesterol,  
12 triglycerides and free fatty acids), obesity, and high blood  
13 pressure (hypertension). Any one of these four first stage  
14 conditions signals the start of Syndrome X.

15 Each first stage Syndrome X condition risks leading to  
16 another. For example, increased insulin production is  
17 associated with high blood fat levels, high blood pressure,  
18 and obesity. Furthermore, the effects of the first stage  
19 conditions are additive; an increase in the number of  
20 conditions causes an increase in the risk of developing more  
21 serious diseases on the Syndrome X continuum.

22 A patient who begins the Syndrome X continuum risks  
23 spiraling into a maze of increasingly deadly diseases. The

1 next stages of the Syndrome X continuum lead to overt  
2 diabetes, kidney failure, and heart failure, with the  
3 possibility of stroke and heart attack at any time. Syndrome  
4 X is a dangerous continuum, and preventative medicine is the  
5 best defense. Diseases are currently most easily diagnosed  
6 in their later stages, but controlling them at a late stage  
7 is extremely difficult. Disease prevention is much more  
8 effective at an earlier stage.

9 In a further contemplated embodiment of the invention,  
10 samples may be taken from a patient at one point in time, as  
11 a single sample or as multiple samples, or at different  
12 points in time such that analysis is carried out on multiple  
13 samples for ongoing analysis. Typically, a first sample is  
14 taken from a patient upon presentation with possible symptoms  
15 of a disease and analyzed according to the invention.  
16 Subsequently, some period of time after presentation, for  
17 example, about 3 - 6 months after the first presentation, a  
18 second sample is taken and analyzed according to the  
19 invention. The data can be used, by way of example, to  
20 diagnose or monitor a disease state, determine risk  
21 assessment, identify therapeutic avenues, or determine the  
22 therapeutic value of an agent such as a pharmaceutical.

23 Subsequent to the isolation of particular disease state

1 marker sequences as taught by the instant invention, the  
2 promulgation of various forms of risk assessment tests are  
3 contemplated which will allow physicians to identify  
4 asymptomatic patients before they suffer an irreversible  
5 event such as diabetes, kidney failure, and heart failure,  
6 and enable effective disease management and preventative  
7 medicine. Additionally, the specific diagnostic tests which  
8 evolve from this methodology provide a tool for rapidly and  
9 accurately diagnosing acute Syndrome X events such as heart  
10 attack and stroke, and facilitate treatment.

11 More particularly, biopolymer markers elucidated via  
12 methodologies of the instant invention find utility related  
13 to broad areas of disease therapeutics. Such therapeutic  
14 avenues include, but are not limited to:

15 1) utilization and recognition of said biopolymer  
16 markers, variants or moieties thereof as direct therapeutic  
17 modalities, either alone or in conjunction with an effective  
18 amount of a pharmaceutically effective carrier;

19 2) validation of therapeutic modalities or disease  
20 preventative agents as a function of biopolymer marker  
21 presence or concentration;

22 3) treatment or prevention of a disease state by  
23 formation of disease intervention modalities; e.g. formation

1 of biopolymer/ligand conjugates which intervene at receptor  
2 sites to prevent, delay or reverse a disease process;

3 4) use of biopolymer markers or moieties thereof as a  
4 means of elucidating therapeutically viable agents, e.g. from  
5 a bacteriophage peptide display library, a bacteriophage  
6 antibody library or the like;

7 5) instigation of a therapeutic immunological  
8 response; and

9 6) synthesis of molecular structures related to said  
10 biopolymer markers, moieties or variants thereof which are  
11 constructed and arranged to therapeutically intervene in the  
12 disease process.

13 A process for identifying or developing therapeutic  
14 avenues related to a disease state utilizing any of the above  
15 examples may follow results obtained from conducting an  
16 analysis inclusive of interacting with a biopolymer including  
17 the sequence of the particular disease specific marker or at  
18 least one analyte thereof of the present invention. Such  
19 treatment or prevention of a disease state by formation of  
20 disease intervention modalities may be by the formation of  
21 biopolymer/ligand conjugates which intervene at receptor  
22 sites to prevent, delay, or reverse a disease process. In  
23 addition, a means of elucidating therapeutically viable

agents may include the use of a bacteriophage peptide display library or a bacteriophage antibody library. The therapeutic avenues may regulate the presence or absence of the biopolymer including the sequence of the particular disease specific marker or at least one analyte thereof in the present invention.

Accordingly, it is an objective of the instant invention to define a disease specific biopolymer marker sequence which is useful in evidencing and categorizing at least one particular disease state.

It is an additional objective of the instant invention to develop methods and means of disease therapy, including but not limited to:

1) utilization and recognition of said biopolymer markers, variants or moieties thereof as direct therapeutic modalities, either alone or in conjunction with an effective amount of a pharmaceutically effective carrier;

2) validation of therapeutic modalities or disease preventative agents as a function of biopolymer marker presence or concentration;

3) treatment or prevention of a disease state by formation of disease intervention modalities; e.g. formation of biopolymer/ligand conjugates which intervene at receptor

1 sites to prevent, delay or reverse a disease process;

2 4) use of biopolymer markers or moieties thereof as a  
3 means of elucidating therapeutically viable agents, e.g. from  
4 a bacteriophage peptide display library, a bacteriophage  
5 antibody library or the like;

6 5) instigation of a therapeutic immunological  
7 response; and

8 6) synthesis of molecular structures related to said  
9 biopolymer markers, moieties or variants thereof which are  
10 constructed and arranged to therapeutically intervene in the  
11 disease process, e.g. by directly determining the three-  
12 dimensional structure of said biopolymer marker directly from  
13 an amino acid sequence thereof.

14 It is another objective of the instant invention to  
15 evaluate samples containing a plurality of biopolymers for  
16 the presence of disease specific biopolymer marker sequences  
17 (disease specific markers) which evidence a link to at least  
18 one specific disease state.

19 It is a further objective of the instant invention to  
20 elucidate essentially all biopolymeric markers, moieties or  
21 variants thereof contained within said samples, whereby  
22 particularly significant moieties may be identified.

23 It is a further objective of the instant invention

1 provide at least one purified antibody which is specific to  
2 said disease specific marker sequence.

3 It is yet another objective of the instant invention to  
4 teach a monoclonal antibody which is specific to said disease  
5 specific marker sequence.

6 It is a still further objective of the invention to  
7 teach polyclonal antibodies raised against said disease  
8 specific marker.

9 It is yet an additional objective of the instant  
10 invention to teach a diagnostic kit for determining the  
11 presence, concentration, or relative strength/concentration  
12 of said disease specific marker.

13 It is a still further objective of the instant invention  
14 to teach methods for characterizing disease state based upon  
15 the identification of said disease specific marker.

16 Other objects and advantages of this invention will  
17 become apparent from the following description taken in  
18 conjunction with the accompanying drawings wherein are set  
19 forth, by way of illustration and example, certain  
20 embodiments of this invention. The drawings constitute a  
21 part of this specification and include exemplary embodiments  
22 of the present invention and illustrate various objects and  
23 features thereof.

1  
2 BRIEF DESCRIPTION OF THE FIGURES

3 Figure 1 is a photograph of a tricine gel DEAE 1 (Elution)  
4 comparing normal human specimen versus Type II diabetes;

5 Figure 2 is a trypsin digested spectra graph depicting the  
6 ion 1628;

7 Figure 3 is a photograph of a tricine gel HiQ3 (scrub)  
8 comparing normal human specimen versus Type II diabetes;

9 Figure 4 is a trypsin digested spectra graph depicting the  
10 ion 1630;

11 Figure 5 is a trypsin digested spectra graph depicting the  
12 ion 1913;

13 Figure 6 is a trypsin digested spectra graph depicting the  
14 ion 1682;

15 Figure 7 is a trypsin digested spectra graph depicting the  
16 ion 2127; and

17 Figure 8 is a trypsin digested spectra graph depicting the  
18 ion 1927.

19  
20 DETAILED DESCRIPTION OF THE INVENTION

21 In earlier work, for example in U.S. Patent application  
22 09/846330 filed April 30, 2000, the contents of which is  
23 herein incorporated by reference, raw sera was obtained and

1 mixed with formic acid and extracted the peptides with C18  
2 reversed phase ZIPTIPs.

3 In the instantly disclosed invention, we deal with  
4 proteins generally having a molecular weight of about 20 kD  
5 or more. In general, proteins of greater than 20 kD can  
6 reliably be fragmented by trypsin or other enzymes. The  
7 instant technology incorporates sufficient sensitivity to  
8 deal with even the low production of peptides from proteins  
9 less than 20 kD clipped from gel.

10 Proteins differ from peptides in that they cannot be  
11 effectively resolved by time of flight MS and they are too  
12 large (>3kD) to be effectively fragmented by collision with  
13 gases. The most commonly used solution to these problems is  
14 to resolve the proteins by polyacrylamide gel electrophoresis  
15 followed by staining with silver, or coomassie brilliant blue  
16 or rubidium dyes or counter staining with Zinc-SDS complexes.  
17 Once the proteins have been resolved and visualized with  
18 stains the proteins that differ between disease states can  
19 then be excised from the gel and the protein purified in the  
20 1-D gel band or 2-D gel spot can be cleaved into fragments  
21 less than 3 kD by proteolytic enzymes. Once protein has been  
22 resolved by gel and cleaved by enzymes, the protein is  
23 considered in the form of peptides and therefore can be dealt

1 with as per earlier work (09/846330). The peptide is either  
2 collected and purified with C18 reversed phase chromatography  
3 or by some other form of chromatography prior to reversed  
4 phase separation. The peptide can also be collected in  
5 ammonium carbonate buffer that is subsequently evolved by  
6 reaction with acid or by removal in organic solvents.

7 Once the peptides are collected they can be sequenced,  
8 e.g. with a MALDI-Qq-TOF but also with a TOF-TOF, and  
9 ESI-Q-TOF or an ION-TRAP. Other types of MS analysis which  
10 may be employed are SELDI MS and MS/MS. The peptides are  
11 fragments of the original protein. The peptides are  
12 sequenced by fragmentation to produced a spectrum composed of  
13 the parts of the peptide. The peptide fragments can be  
14 produced by a strong ionization energy with a laser,  
15 temperature, electron capture, collision between the peptides  
16 themselves or with other objects such as gas molecules. The  
17 spacing in terms of mass between the parts of the peptides is  
18 a fragmentation pattern. The fragmentation pattern of each  
19 peptide from the starting mass to the last remaining amino  
20 acid (from either end) is unique.

21 The human genome contains the genes that encode all  
22 proteins. The proteolytic cut sites within all these  
23 proteins can be predicted from the translated amino acid

1 sequence. The mass of the peptides that result from the  
2 predicting cut sites can be calculated. Similarly, the  
3 fragmentation pattern from each hypothetical peptide can be  
4 predicted. Thus, we can conceptually digest the proteins  
5 within the human proteome and fragment them.

6 When a peptide has been "sequenced" it is understood  
7 that the peptide fragment has been purified by one of the  
8 methods above, i.e. Time of flight (TOF) or by  
9 chromatography, before fragmenting it with gas to produce the  
10 peptide fragments. The original peptide mass and  
11 fragmentation pattern obtained is then fit to those from the  
12 theoretical digestion and fragmentation of the genome. The  
13 peptide that best matches the theoretical peptides and  
14 fragments and is biologically possible, i.e. a potential  
15 human blood-borne protein, is thus identified. It is possible  
16 to identify plural targets in this fashion.

17  
18 Following are exemplary, but non-limiting examples of  
19 preparatory protocols useful in the process of the instant  
20 invention.

21  
22 Preparatory Protocols:

23 Any of these protocols may be selected from a column

1 flow-through stream, a column elution stream, or a column  
2 scrub stream.

3 Hi Q is a strong anion exchanger made of methyl acrylate  
4 co-polymer with the functional group:  $-N^+(CH_3)_2$ ;

5 Hi S is a strong cation exchanger made of methyl acrylate  
6 co-polymer with the functional group:  $-SO_3^-$ ;

7 DEAE is diethylaminoethyl which is a weak cation exchanger  
8 made of methyl acrylate co-polymer with the functional group  
9  $-N^+(C_2H_5)_2$ ;

10 PS is phenyl sepharose;

11 BS is butyl sepharose.

12 Note that the supports, i.e. methyl acrylate and  
13 sepharose are different, but non-limiting examples, as the  
14 same functional group on different supports will function,  
15 albeit possibly with different effects.

16 DEAE Column Protocol:

- 17 1) Cast 200  $\mu$ l of 50% slurry;
- 18 2) Equilibrate column in 5 bed volumes of 50 mM  
19 tricine pH 8.8 (binding buffer);
- 20 3) Dissolve 25  $\mu$ l of sera in 475  $\mu$ l of binding buffer;
- 21 4) Wash column in 5 bed volumes of binding buffer;
- 22 5) Elute column in 120  $\mu$ l of 0.4 M Phosphate buffer  
23 (PB) pH 6.1;

- 1 6) Elute column in 120 µl of 50 mM citrate buffer  
2 pH 4.2;  
3 7) Scrub column with 120 µl sequentially with each  
4 of 0.1% triton, 1.0% triton and 2% SDS in  
5 62.5 mM Tris pH 6.8.  
6

7 Butyl Sepharose Column Protocol:

- 8 1) Cast 150 µl bed volume column;  
9 2) Equilibrate column in 5 bed volumes of 1.7 M  
10  $(\text{NH}_4)_2\text{SO}_4$  in 50 mM PB pH 7.0 (binding buffer);  
11 3) Dissolve 35 µl of sera in 465 µl of binding buffer  
12 and apply;  
13 4) Wash column in 5 bed volumes of binding buffer;  
14 5) Elute column in 120 µl of 0.4 M  $(\text{NH}_4)_2\text{SO}_4$  in  
15 50 mM PB pH 7.0;  
16 6) Elute column in 120 µl of 50 mM PB pH 7.0;  
17 7) Scrub column with 120 µl sequentially with each  
18 of 0.1% triton, 1.0% triton and 2% SDS in  
19 62.5 mM Tris pH 6.8.  
20  
21

22 Phenyl Sepharose Column Protocol:

- 23 1) Cast 150 µl bed volume column;

- 1 2) Equilibrate column in 5 bed volumes of  
2 1.7 M  $(\text{NH}_4)_2\text{SO}_4$  in 50 mM PB pH 7.0 (binding buffer);  
3 3) Dissolve 35  $\mu\text{l}$  of sera in 465  $\mu\text{l}$  of binding  
4 buffer and apply;  
5 4) Wash column in 5 bed volumes of binding buffer;  
6 5) Elute column in 120  $\mu\text{l}$  of 0.2 M  $(\text{NH}_4)_2\text{SO}_4$  in  
7 50 mM PB pH 7.0;  
8 6) Elute column in 120  $\mu\text{l}$  of 50 mM PB pH 7.0;  
9 7) Scrub column with 120  $\mu\text{l}$  sequentially with each  
10 of 0.1% triton, 1.0% triton and 2% SDS in  
11 62.5 mM Tris pH 6.8.

12  
13 HiQ Anion Exchange Mini Column Protocol:

- 14 1) Dilute sera in sample/running buffer;  
15 2) Add HiQ resin to column and remove any air bubbles;  
16 3) Add ultrafiltered (UF) water to aid in column  
17 packing;  
18 4) Add sample/running buffer to equilibrate column;  
19 5) Add diluted sera;  
20 6) Collect all the flow-through fraction in Eppendorf  
21 tubes until level is at resin;  
22 7) Add sample/running buffer to wash column;  
23 8) Add elution buffer and collect elution in Eppendorf

1 tubes.

2

3 HiS Cation Exchange Mini Column Protocol:

4 1) Dilute sera in sample/running buffer;

5 2) Add HiS resin to column and remove any air bubbles;

6 3) Add UF water to aid in column packing;

7 4) Add sample/running buffer to equilibrate column for  
8 sample loading;

9 5) Add diluted sera to column;

10 6) Collect all flow through fractions in Eppendorf  
11 tubes until level is at resin;

12 7) Add sample/running buffer to wash column;

13 8) Add elution buffer and collect elution in Eppendorf  
14 tubes.

15 Illustrative of the various buffering compositions  
16 useful in this technique are:

17 Sample/Running buffers: including but not limited to

18 Bicine buffers of various molarities, pH's, NaCl content,

19 Bis-Tris buffers of various molarities, pH's, NaCl

20 content, Diethanolamine of various molarities, pH's, NaCl

21 content, Diethylamine of various molarities, pH's, NaCl

22 content, Imidazole of various molarities, pH's, NaCl

23 content, Tricine of various molarities, pH's, NaCl

1 content, Triethanolamine of various molarities, pH's, NaCl  
2 content, Tris of various molarities, pH's, NaCl content.  
3 Elution Buffer: Acetic acid of various molarities, pH's,  
4 NaCl content, Citric acid of various molarities, pH's,  
5 NaCl content, HEPES of various molarities, pH's, NaCl  
6 content, MES of various molarities, pH's, NaCl content,  
7 MOPS of various molarities, pH's, NaCl content, PIPES of  
8 various molarities, pH's, NaCl content, Lactic acid of  
9 various molarities, pH's, NaCl content, Phosphate of  
10 various molarities, pH's, NaCl content, Tricine of various  
11 molarities, pH's, NaCl content.

12 Following tryptic digestion, additional processing  
13 may be carried out, for example:  
14 Utilizing a type of micro-chromatographic column called a  
15 C18- ZIPTIP available from the Millipore company, the  
16 following preparatory steps were conducted.

- 17 1. Dilute sera in sample buffer
- 18 2. Aspirate and dispense ZIPTIP in 50% Acetonitrile
- 19 3. Aspirate and dispense ZIPTIP in Equilibration solution
- 20 4. Aspirate and dispense in serum sample
- 21 5. Aspirate and dispense ZIPTIP in Wash solution
- 22 6. Aspirate and dispense ZIPTIP in Elution Solution

23 Illustrative of the various buffering compositions

1 useful in the present invention are:

2 Sample Buffers (various low pH's): Hydrochloric acid  
3 (HCl), Formic acid, Trifluoroacetic acid (TFA),

4 Equilibration Buffers (various low pH's): HCl, Formic  
5 acid, TFA;

6 Wash Buffers (various low pH's): HCl, Formic acid, TFA;

7 Elution Solutions (various low pH's and % Solvents):

8 HCl, Formic acid, TFA;

9 Solvents: Ethanol, Methanol, Acetonitrile.

10 Spotting was then performed, for example upon a Gold Chip  
11 in the following manner:

12 1. Spot 2 ul of sample onto each spot

13 2. Let sample partially dry

14 As a result of these procedures, the disease specific  
15 markers namely Fibronectin Precursors having a molecular  
16 weight of about 1629.94 daltons and a sequence of  
17 VDVIPVNLPGHEHGQR, a molecular weight of about 1927.0442  
18 daltons and a sequence of (R)FLATTPNSLLVSWQPPR(A), a  
19 molecular weight of about 2127 daltons and a sequence of  
20 HQLYIDETVNSNIPTNLR, a molecular weight of about 1629.87  
21 daltons and a sequence of RVDVIPVNLPGHEHGQRL, a molecular  
22 weight of about 1913.08 daltons and a sequence of  
23 SSPVVIDASTAIDAPSNLR, and a molecular weight of about

1 1682.96 daltons having a sequence of IHLISTQSAIPYALR  
2 related to Type II diabetes were found.

3 Figures 1 and 3 are photographs of a gel which is  
4 indicative of the presence/absence of the marker in disease  
5 vs. control and, in cases where the marker is always  
6 present, the relative strength, e.g. the up or down  
7 regulation of the marker relative to categorization of  
8 disease state is deduced.

9 A method for evidencing and categorizing at least one  
10 disease state is disclosed. The steps taken include  
11 obtaining a sample from a patient, preferably human, and  
12 conducting MS analysis on the sample. As a result, at least  
13 one biopolymer marker sequence or analyte thereof is  
14 isolated from the sample which undergoes evidencing and  
15 categorizing and is compared to the biopolymer marker  
16 sequence as disclosed in the present invention. The step of  
17 evidencing and categorizing is particularly directed to  
18 biopolymer markers or analytes thereof linked to at least  
19 one risk of disease development of the patient or related to  
20 the existence of a particular disease state.

21 In addition, various kits are contemplated for use by  
22 the present invention. One such kit provides for  
23 determining the presence of the disease specific biopolymer

1 marker. At least one biochemical material is incorporated  
2 which is capable of specifically binding with a biomolecule  
3 which includes at least the disease specific biopolymer  
4 marker or analyte thereof, and a means for determining  
5 binding between the biochemical material and the  
6 biomolecule. The biochemical material for any of the  
7 contemplated kits, by way of example an antibody or at least  
8 one monoclonal antibody specific therefore, or biomolecule  
9 may be immobilized on a solid support and include at least  
10 one labeled biochemical material which is preferably an  
11 antibody. The sample utilized for any of the kits may be a  
12 fractionated or unfractionated body fluid or a tissue  
13 sample. Non-limiting examples of such fluids are blood,  
14 blood products, urine, saliva, cerebrospinal fluid, and  
15 lymph.

16 Further contemplated is a kit for diagnosing,  
17 determining risk-assessment, and identifying therapeutic  
18 avenues related to a disease state. This kit includes at  
19 least one biochemical material which is capable of  
20 specifically binding with a biomolecule which includes at  
21 least one biopolymer marker including the sequence of the  
22 particular disease specific biopolymer marker or an  
23 analyte thereof related to the disease state. Also

1 included is a means for determining binding between the  
2 biochemical material and the biomolecule, whereby at least  
3 one analysis to determine a presence of a marker, analyte  
4 thereof, or a biochemical material specific thereto, is  
5 carried out on a sample. As previously described,  
6 analysis may be carried out on a single sample or multiple  
7 samples.

8 In accordance with various stated objectives of the  
9 invention, the skilled artisan, in possession of the  
10 specific disease specific marker as instantly disclosed,  
11 would readily carry out known techniques in order to raise  
12 purified biochemical materials, e.g. monoclonal and/or  
13 polyclonal antibodies, which are useful in the production of  
14 methods and devices useful as point-of-care rapid assay  
15 diagnostic or risk assessment devices as are known in the  
16 art.

17 The specific disease markers which are analyzed  
18 according to the method of the invention are released into  
19 the circulation and may be present in the blood or in any  
20 blood product, for example plasma, serum, cytolyzed blood,  
21 e.g. by treatment with hypotonic buffer or detergents and  
22 dilutions and preparations thereof, and other body fluids,  
23 e.g. CSF, saliva, urine, lymph, and the like. The

1 presence of each marker is determined using antibodies  
2 specific for each of the markers and detecting specific  
3 binding of each antibody to its respective marker. Any  
4 suitable direct or indirect assay method may be used to  
5 determine the level of each of the specific markers  
6 measured according to the invention. The assays may be  
7 competitive assays, sandwich assays, and the label may be  
8 selected from the group of well-known labels such as  
9 radioimmunoassay, fluorescent or chemiluminescence  
10 immunoassay, or immunoPCR technology. Extensive discussion  
11 of the known immunoassay techniques is not required here  
12 since these are known to those of skilled in the art. See  
13 Takahashi et al. (Clin Chem 1999;45(8):1307) for a  
14 detailed example of an assay.

15 A monoclonal antibody specific against the disease  
16 marker sequence isolated by the present invention may be  
17 produced, for example, by the polyethylene glycol (PEG)  
18 mediated cell fusion method, in a manner well-known in the  
19 art.

20 Traditionally, monoclonal antibodies have been made  
21 according to fundamental principles laid down by Kohler  
22 and Milstein. Mice are immunized with antigens, with or  
23 without, adjuvants. The splenocytes are harvested from

1 the spleen for fusion with immortalized hybridoma  
2 partners. These are seeded into microtiter plates where  
3 they can secrete antibodies into the supernatant that is  
4 used for cell culture. To select from the hybridomas that  
5 have been plated for the ones that produce antibodies of  
6 interest, the hybridoma supernatants are usually tested  
7 for antibody binding to antigens in an ELISA (enzyme  
8 linked immunosorbent assay) assay. The idea is that the  
9 wells that contain the hybridoma of interest will contain  
10 antibodies that will bind most avidly to the test antigen,  
11 usually the immunizing antigen. These wells are then  
12 subcloned in limiting dilution fashion to produce  
13 monoclonal hybridomas. The selection for the clones of  
14 interest is repeated using an ELISA assay to test for  
15 antibody binding. Therefore, the principle that has been  
16 propagated is that in the production of monoclonal  
17 antibodies the hybridomas that produce the most avidly  
18 binding antibodies are the ones that are selected from  
19 among all the hybridomas that were initially produced.  
20 That is to say, the preferred antibody is the one with  
21 highest affinity for the antigen of interest.

22 There have been many modifications of this procedure  
23 such as using whole cells for immunization. In this

1 method, instead of using purified antigens, entire cells  
2 are used for immunization. Another modification is the  
3 use of cellular ELISA for screening. In this method  
4 instead of using purified antigens as the target in the  
5 ELISA, fixed cells are used. In addition to ELISA tests,  
6 complement mediated cytotoxicity assays have also been  
7 used in the screening process. However, antibody-binding  
8 assays were used in conjunction with cytotoxicity tests.  
9 Thus, despite many modifications, the process of producing  
10 monoclonal antibodies relies on antibody binding to the  
11 test antigen as an endpoint.

12 The purified monoclonal antibody is utilized for  
13 immunochemical studies.

14 Polyclonal antibody production and purification  
15 utilizing one or more animal hosts in a manner well-known  
16 in the art can be performed by a skilled artisan.

17 Another objective of the present invention is to  
18 provide reagents for use in diagnostic assays for the  
19 detection of the particularly isolated disease specific  
20 marker sequences of the present invention.

21 In one mode of this embodiment, the marker sequences  
22 of the present invention may be used as antigens in  
23 immunoassays for the detection of those individuals

1 suffering from the disease known to be evidenced by said  
2 marker sequence. Such assays may include but are not  
3 limited to: radioimmunoassay, enzyme-linked immunosorbent  
4 assay (ELISA), "sandwich" assays, precipitin reactions,  
5 gel diffusion immunodiffusion assay, agglutination assay,  
6 fluorescent immunoassays, protein A or G immunoassays and  
7 immunoelectrophoresis assays.

8 According to the present invention, monoclonal or  
9 polyclonal antibodies produced against the disease  
10 specific marker sequence of the instant invention are  
11 useful in an immunoassay on samples of blood or blood  
12 products such as serum, plasma or the like, cerebrospinal  
13 fluid or other body fluid, e.g. saliva, urine, lymph, and  
14 the like, to diagnose patients with the characteristic  
15 disease state linked to said marker sequence. The  
16 antibodies can be used in any type of immunoassay. This  
17 includes both the two-site sandwich assay and the single  
18 site immunoassay of the non-competitive type, as well as  
19 in traditional competitive binding assays.

20 Particularly preferred, for ease and simplicity of  
21 detection, and its quantitative nature, is the sandwich or  
22 double antibody assay of which a number of variations  
23 exist, all of which are contemplated by the present

1 invention. For example, in a typical sandwich assay,  
2 unlabeled antibody is immobilized on a solid phase, e.g.  
3 microtiter plate, and the sample to be tested is added.  
4 After a certain period of incubation to allow formation of  
5 an antibody-antigen complex, a second antibody, labeled  
6 with a reporter molecule capable of inducing a detectable  
7 signal, is added and incubation is continued to allow  
8 sufficient time for binding with the antigen at a  
9 different site, resulting with a formation of a complex of  
10 antibody-antigen-labeled antibody. The presence of the  
11 antigen is determined by observation of a signal which may  
12 be quantitated by comparison with control samples  
13 containing known amounts of antigen.

14 Antibodies may also be utilized against the disease  
15 specific markers, as haptens, to create an antibody  
16 response against the protein to which it binds, thereby  
17 identifying targets for treatment of the disease or a sub-  
18 class thereof.

19 Lastly, the markers and associated antibodies provide  
20 a tool for monitoring the progress of a patient during a  
21 therapeutic treatment, so as to determine the usefulness  
22 of a novel therapeutic agent.

23 All patents and publications mentioned in this

1 specification are indicative of the levels of those  
2 skilled in the art to which the invention pertains. All  
3 patents and publications are herein incorporated by  
4 reference to the same extent as if each individual  
5 publication was specifically and individually indicated to  
6 be incorporated by reference.

7 It is to be understood that while a certain form of  
8 the invention is illustrated, it is not to be limited to  
9 the specific form or arrangement herein described and  
10 shown. It will be apparent to those skilled in the art  
11 that various changes may be made without departing from  
12 the scope of the invention and the invention is not to be  
13 considered limited to what is shown and described in the  
14 specification and drawings/figures.

15 One skilled in the art will readily appreciate that  
16 the present invention is well adapted to carry out the  
17 objectives and obtain the ends and advantages mentioned,  
18 as well as those inherent therein. The oligonucleotides,  
19 peptides, polypeptides, biologically related compounds,  
20 methods, procedures and techniques described herein are  
21 presently representative of the preferred embodiments, are  
22 intended to be exemplary and are not intended as  
23 limitations on the scope. Changes therein and other uses

1 will occur to those skilled in the art which are  
2 encompassed within the spirit of the invention and are  
3 defined by the scope of the appended claims. Although the  
4 invention has been described in connection with specific  
5 preferred embodiments, it should be understood that the  
6 invention as claimed should not be unduly limited to such  
7 specific embodiments. Indeed, various modifications of the  
8 described modes for carrying out the invention which are  
9 obvious to those skilled in the art are intended to be  
10 within the scope of the following claims.  
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